

Analysis of Multiple *Plasmodium falciparum* Infections in Tanzanian Children during the Phase III Trial of the Malaria Vaccine SPf66

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In the first phase III efficacy trial of the malaria vaccine SPf66 in Africa, MOIs in SPf66- and placebo-vaccinated children were analyzed by polymerase chain reaction–restriction fragment length polymorphism of the *Plasmodium falciparum* merozoite surface antigen 2 (MSA2). MOIs were significantly reduced in asymptomatic vaccine recipients compared with those in asymptomatic placebo recipients; however, no differences were observed among symptomatic children in the vaccine and control groups. These results show that immunization with SPf66 modulates the course of naturally occurring infections, as reflected by reduced MOIs. In placebo recipients, however, there was a significant negative correlation between numbers of infecting genotypes, as identified by MSA2, and morbidity. Asymptomatic placebo recipients had an average of 5 concurrent infections, whereas children with clinical cases had an average of 3.4 infections. These data provide further evidence that premunition from concurrent infections is important in immunity against clinical malaria. No such effect of multiple infections was found in the vaccinated group.

The malaria vaccine SPf66 has been extensively tested in South American populations, with a variable degree of efficacy against clinical *Plasmodium falciparum* malaria [1]. In phase III trials against clinical malaria, the vaccine had an efficacy of 39% in Colombia [2], 55% in Venezuela [3], and 66% in Ecuador [4], three countries with low malaria endemicity. Recently, SPf66 was tested in children and infants in malaria-endemic areas of Africa. The vaccine had an estimated protective efficacy of 31% against clinical episodes in 1- to 5-year-old Tanzanian children in an area where malaria transmission is intense and perennial [5]. In The Gambia, no significant protection was observed in a trial involving infants who were 6–11 months old at the time of the first vaccine dose and who were exposed to seasonal malaria [6]. The African trials, however, were not completely comparable, primarily because of the different endemicity situations but also due to trial designs [6]. A recent SPf66 trial in northwestern Thailand also showed no vaccine efficacy [7].

To analyze the effect of vaccination with SPf66 on parasite diversity and MOI, we genotyped *P. falciparum* infections from vaccine and placebo recipients from the Tanzanian trial for the merozoite surface antigen 2 (MSA2). MSA2 has been shown

to be highly polymorphic, yet all identified alleles could be grouped into the two allelic families, FC27 and 3D7 [8].

MSA2 is a useful marker in studies of diversity in *Plasmodium* populations and has been used in several other studies in an attempt to describe parasite diversity within endemic populations [9–12]. We recently reported different age distributions of the two allelic families of MSA2 in a malaria-exposed population in Papua New Guinea [10]. However, since multiple infections are relatively infrequent in Papua New Guinea, only limited investigation of the effects of MOIs on parasite density and morbidity was possible.

From studies conducted in areas with different endemicity, it appears that numbers and multiplicity of concurrent *P. falciparum* infections as determined by MSA2 analysis could be markers for exposure [10–13]. In hyperendemic areas, such as Senegal or Tanzania, multiple infections have been observed with high frequencies, and MOIs were high [13, 14]. In holoendemic areas, such as lowland areas of Papua New Guinea, multiple infections were observed less frequently, and multiplicity was low [10, 15, 16]. In this study, we investigated *P. falciparum* MOIs in vaccine and placebo recipients.

Material and Methods

Samples. Samples described in this study were collected during a phase III randomized trial of the SPf66 malaria vaccine in Idete village, Kilombero District, Tanzania [5]. Details of the trial design have been presented elsewhere [17]. In brief, children who were 1 to 5 years old at the time of the first dose of placebo or vaccine were treated with sulfadoxine-pyrimethamine (Fansidar; Roche, Basel, Switzerland) to clear parasitemia at each of the three immunizations. Reinfection after treatment was followed at repeated cross-sectional surveys in a subgroup of children, and clinical malaria was monitored by case detection at the Idete dispensary [5, 18]. This study concerns only samples collected subse-

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All experiments involving humans followed the guidelines of conduct of clinical research and were performed after informed consent of the children's parents or guardians. Research and ethical clearance was granted by the Tanzanian Commission for Science and Technology (NSR/RCA 90).

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quent to the third dose of vaccine or placebo at week 26 of the trial protocol.

Clinical malaria cases. Clinical malaria was defined as axillary temperature of $\geq 37.5^{\circ}\text{C}$ and a density of asexual parasites of $>5000/\mu\text{L}$ by conventional light microscopy [5]. This parasite density cutoff was lower than that used in the primary estimates of vaccine efficacy [5]. This less-specific case definition was chosen for the present analyses because insufficient samples with parasite densities of $>20,000/\mu\text{L}$ were available from vaccine recipients with clinical episodes [17].

For each vaccine recipient who provided ≥ 1 sample during a clinical malaria episode, only 1 such sample was selected randomly and included in the present study. These samples were stratified according to the nearest, in time, cross-sectional survey (at week 30, 50, or 73 of the protocol [17]). From each of these time periods, samples from clinical episodes of placebo recipients were also included. Again, only 1 sample (chosen at random within the above constraints) from each child was analyzed.

Community controls. Control samples were selected from cross-sectional surveys done as part of the vaccine trial. Only samples positive for asexual-stage *P. falciparum* by microscopy and only those from children who were not represented among the cases were considered. The control samples were chosen to ensure equal representation of each time period in cases and controls for both placebo and vaccine recipients. Insofar as this was possible, controls were also selected to have the same age distributions as cases. However, because clinical malaria occurred predominantly in the youngest children [19], it was not possible to select enough control samples from children who were as young as the cases; therefore, some samples from older children were tested. Only 1 sample was included from each child.

Laboratory methods. For genotyping, DNA was isolated from 5 μL of blood in 4 M guanidine isothiocyanate containing 25 mM sodium-citrate, 0.2 M sodium-acetate, pH 4.4, 0.5% SDS, and 0.2 M β -mercaptoethanol. The mixture was phenol-treated once and precipitated with isopropanol. For MSA2 genotyping, one-tenth of the DNA was amplified by polymerase chain reaction (PCR), using the primers S2/S3 and S1/S4 for primary and nested PCR, respectively [20]. The PCR products subsequently were subjected to an array of restriction enzymes, and the resulting fragments were separated on 12% polyacrylamide gels as described [21]. Figure 1 is an example of the restriction fragment length polymorphism (RFLP) pattern of 10 concurrent infections found in 1 sample. RFLP patterns were assessed visually, and infections were enumerated independently by 3 researchers blinded to the source of the samples. Samples with inconsistent results were repeated and read again.

Results

In total, 282 samples were genotyped for MSA2. Table 1 provides demographic and parasitologic data for the children included in this analysis.

MSA2 was genotyped by PCR-RFLP in 135 samples from vaccinated children and in 147 samples from placebo recipients. After treatment with sulfadoxine-pyrimethamine, which was required in the trial design, the MOIs showed a very rapid

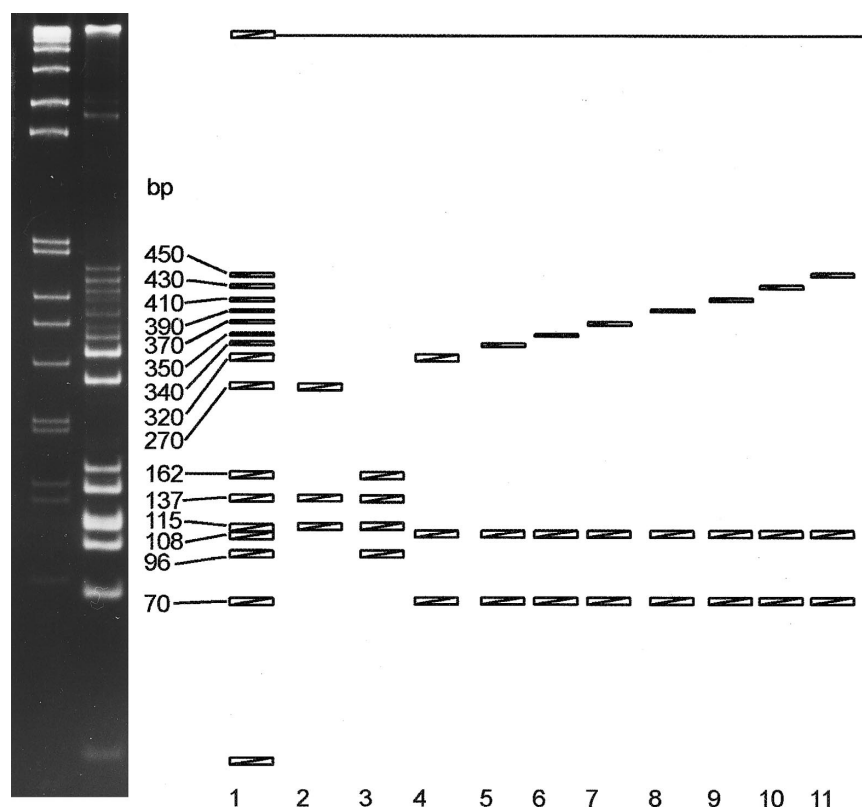
increase with time. At week 30, 2 asymptomatic placebo recipients had a mean MOI of only 1.5 (SE = 0.5), but by week 50 it had increased to 4.5 (SE = 0.4), and at week 73, the average number of MSA2 genotypes per sample in placebo recipients was 5.5. In clinical malaria cases, there was little difference between surveys (data not shown). There was a positive correlation in asymptomatic vaccinated children with the elapsed time after sulfadoxine-pyrimethamine treatment and the number of infections (Spearman's rank correlation [r_s] = .52; $P < .001$). In all 4 groups, there was no statistically significant age-dependency of multiplicity when corrected for elapsed time after treatment (for asymptomatic placebo recipients, partial r_s = -.09; for asymptomatic vaccine recipients, partial r_s = .09; for symptomatic placebo recipients, partial r_s = .07; and for symptomatic vaccinated children, partial r_s = -.18). In symptomatic cases in both vaccine and placebo recipients, multiplicity was completely independent of the elapsed time after treatment.

The frequency and distribution of multiple MSA2 infections is shown in table 2. PCR-RFLP analysis of MSA2 not only allowed enumeration of multiple infections but also identification of the two allelic families (FC27 or 3D7) [8] to which the individual alleles belonged (table 2). Twelve crossing-overs between MSA2 alleles of different allelic families as described by Ntoumi et al. [13] and Marshall et al. [22] were detected by recombination of the family-specific restriction fragments. These alleles are currently being analyzed by sequencing. For classification of allelic families, we used the immunologically important repeat region, and all genotypes containing the FC27-like 32-aa repeat were categorized as an FC27-like genotype.

The comparative analysis of numbers of infections in asymptomatic vaccine recipients and healthy placebo recipients showed a significant reduction of MOIs. In vaccinated children, the mean number of concurrent infections was 3.9/sample, and in placebo recipients it was 5.0/sample ($P = .005$, Wilcoxon $Z = 2.84$). A reduction was also seen for the individual allelic families (table 2). In contrast, in clinical cases the mean number of infections was similar in the vaccinated group and in the placebo recipients (table 2). In neither group were levels of parasitemia correlated with the overall MOIs, but a significant correlation with the number of FC27-like infections in asymptomatic vaccinees was observed ($r_s = 0.27$, $P = .02$). A similar correlation was found in the placebo recipients ($r_s = 0.32$, $P = .005$). There was no association with 3D7-like infections and densities in asymptomatic vaccinees or placebo recipients, suggesting that these infections generally made only a small contribution to the overall density. These findings were not significantly affected by adjustment for age of the child or for the survey at which the sample was collected.

When asymptomatic and symptomatic placebo recipients were compared, however, a strong negative association between risk of clinical malaria and the numbers of simultaneously infecting different parasites was observed, a finding that could not be accounted for by age or time trends. A logistic

Figure 1. Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis of multiple MSA2 infections in 1 blood sample of asymptomatic child, showing RFLP pattern after *Hinf*I digest (right lane of gel). Left lane of gel is 1-kb ladder (GIBCO BRL, Eggenstein, Germany). Lane 1: graphic representation of complete RFLP pattern; lanes 2, 3: individual RFLP pattern of FC27-like genotypes (D10, WOS12); lanes 4–11: 3D7-like genotypes with characteristic 70- and 108-bp fragments.



regression model was used to estimate the risk of being clinically ill with malaria with multiple simultaneous infections. Curves were fitted for all MSA2 genotypes, for 3D7-like infections only, and for FC27-like genotypes only (figure 2). The similar gradients of the curves suggested that multiplicity, not any allelic family of the merozoite surface antigen, confers protection. However, the statistical significance of the regression was greater for 3D7-like genotypes (likelihood ratio $\chi^2 = 18.7$, $P < .001$), due to the considerably larger diversity of this allelic family, than for FC27-like genotypes ($\chi^2 = 6.8$, $P = .009$), which displayed a smaller diversity.

In contrast, no significant difference in numbers of infections was observed among symptomatic and asymptomatic vaccinated children (table 2), and the risk of clinical episodes did not vary with the number of simultaneous infections, as determined by MSA2 PCR (logistic regression: likelihood ratio $\chi^2 = 0.29$, $P = .59$) (figure 2).

Discussion

For the present study, 282 children who were 1–5 years old at the beginning of the study and living in an area of intense

Table 1. Demographic and parasitologic data for malaria-infected study children.

	Placebo recipients		Vaccinated children	
	Asymptomatic (n = 76)	Symptomatic (n = 71)	Asymptomatic (n = 70)	Symptomatic (n = 65)
Mean age, years (\pm SD)	5.0 (1.5)	3.8 (1.2)	4.7 (1.5)	3.8 (1.4)
Mean elapsed time, days (\pm SD), after 3rd sulphadoxine-pyrimethamine treatments	286 (87)	282 (73)	276 (98)	284 (63)
Geometric mean <i>P. falciparum</i> density (95% confidence interval)	3100* (2200–4300)	26,700† (21,000–34,200)	1600* (1100–2200)	17,500† (14,400–23,900)

NOTE. Children randomly selected from each group of phase III trial [5]. For symptomatic children, clinical malaria was defined as axillary temperature $\geq 37^\circ\text{C}$ and >5000 asexual parasites/ μL of blood.

* Significantly different, Wilcoxon $Z = 2.66$, $P = .008$.

† Significantly different, Wilcoxon $Z = 2.16$, $P = .031$.

Table 2. Mean nos. of simultaneous malaria infections in vaccine and placebo recipients by morbidity.

Infection	Placebo recipients, mean (SE)	Vaccine recipients, mean (SE)	Z*	P
MSA2				
Asymptomatic children	4.97 (0.25)	3.90 (0.27)	2.84	.005
Symptomatic children	3.35 (0.25)	3.85 (0.27)	1.43	.15
FC27-like				
Asymptomatic children	2.08 (0.15)	1.60 (0.14)	2.28	.02
Symptomatic children	1.55 (0.14)	1.66 (0.16)	0.50	.6
3D7-like				
Asymptomatic children	2.89 (0.17)	2.30 (0.19)	2.46	.014
Symptomatic children	1.82 (0.18)	2.19 (0.18)	1.82	.07

NOTE. Clinical malaria was defined as axillary temperature $\geq 37^{\circ}\text{C}$ and >5000 asexual parasites/ μL of blood.

* Testing difference between vaccine and placebo recipients (Wilcoxon test).

malaria transmission were analyzed for infecting parasite genotypes. Infections with an average of 5 different genotypes were identified in asymptomatic placebo recipients, using highly sensitive PCR-RFLP for MSA2. In contrast, infections with only 3.9 MSA2 genotypes were identified in asymptomatic vaccine recipients. These data confirm previous results demonstrating

a high multiplicity of *P. falciparum* infections in this area [14]. Similar MOIs determined by use of a different technique were recently reported from Senegal [12, 13]. The MOIs and frequency of multiple infections were much higher than those in studies conducted in Papua New Guinea using the identical method [10, 15], likely as a consequence of the higher exposure

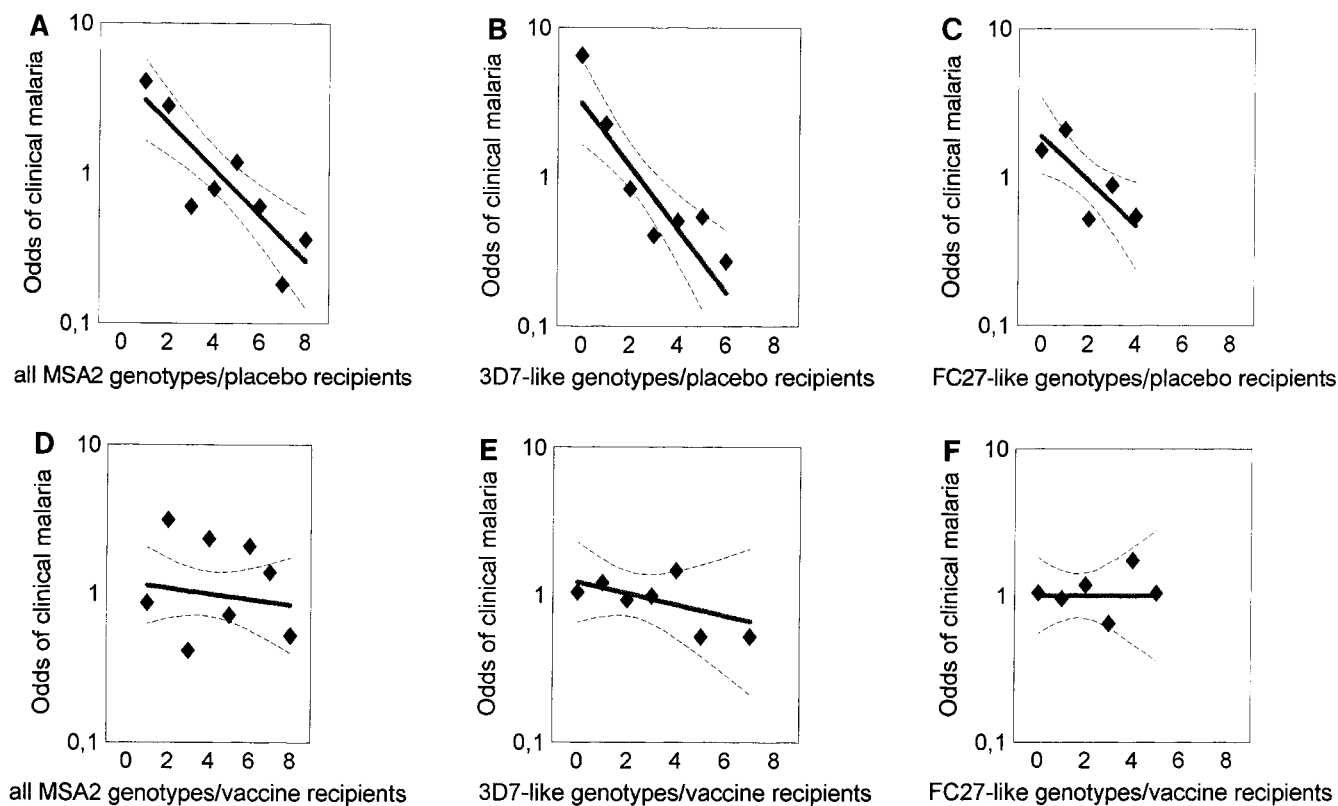


Figure 2. Logistic regression line for risk of clinical malaria in relation to MOI. Observed odds relative to average MOI (◆); fitted logistic regression (solid line); 95% confidence limits (dashed lines). Odds ratios for placebo recipients with all multiple MSA2 infections (A), all 3D7-like genotype infections (B), and all FC27-like genotype infections (C). Odds ratios for vaccine recipients within all multiple MSA2 infections (D), all 3D7-like genotype infections (E), and all FC27-like genotype infections (F).

to malaria in the Kilombero valley than in the study sites in Papua New Guinea [23].

In vaccinated children, MOIs were significantly reduced. The reduction of multiplicity in asymptomatic *P. falciparum* infections associated with SPf66 vaccination seems to be comparable in magnitude to the effects on clinical episodes [5] and on parasite densities [19]. There was no reduction in the incidence of blood-stage infection after three doses of vaccine [5], and SPf66 did not significantly reduce the overall prevalence of infection in the Tanzanian trial [19]. Hence, the reduced MOI in asymptomatic infections is likely to be a consequence of an increased rate of elimination of individual infections. This would correspond to the reported results of the early SPf66 challenge trials [24, 25] in nonimmune hosts, which indicated that SPf66 does not induce sterilizing immunity against *P. falciparum* but that some infections can be self-limiting. It appears that this course may also be followed by individual infections in children with considerable previous exposure and with multiple infections of parasites with different genotypes.

The observed differences in multiplicity between vaccine and placebo recipients may be explained by an immune modulation due to vaccination. In the absence of vaccination, low-density malaria infections can persist because natural immunity to asexual stage parasites appears to be highly density-dependent [23, 24]: The immune system is predominantly stimulated by high-density infections, and low-grade parasitemia may be tolerated. In semi-immune persons, the densities of many infections may thus be reduced to a low level, but infection is not completely eliminated. Unlike naturally stimulated immunity, vaccine-induced immunity is in general not density-dependent (i.e., the primary response does not depend on the intensity or even on the existence of an infection). Our results with vaccine recipients suggest that SPf66 may induce a response sufficient to control high-density infections and hence to prevent morbidity while eliminating low-grade infections. This could result in the observed reduction of MOIs in both groups.

The significant negative correlation between MOIs and morbidity in placebo recipients is in agreement with findings from Papua New Guinea, where multiple infections were associated with a reduced risk of clinical malaria [15]. One explanation for these findings could be that in clinical malaria episodes, the dominant parasite genotype may stimulate a complex cascade of nonspecific responses, such as cytokine-mediated fever and release of nitric oxide or oxygen radicals, which have an antiparasitic effect [26, 27], eliminating nondominant genotypes or suppressing them below the PCR detection limit. This would result in a significantly reduced MOI. Reduced multiplicity in children with clinical episodes could have also been due to more frequent treatments than in controls. But these explanations seem to be unlikely because no such effect could be observed in the vaccine recipients. The difference in times since last treatment was similar for controls and cases, and no reduction of numbers of infections was observed in clinical case-patients.

An alternative explanation for the observed negative correlation of multiple parasite infections with morbidity in nonvaccinated children is that a high MOI provides protection per se. In support of our interpretation, a recent study in Papua New Guinea, in which the prospective risk of clinical malaria was analyzed in relation to multiple infections, showed that children with multiple infections had a lower prospective risk of clinical malaria (Al-Yaman F, personal communication). There is evidence that clinical malaria in children in endemic areas occurs as a result of acquisition of a parasite with an antigen genotype to which they do not yet have immunity [16, 28]. As a child ages, the repertoire of genotypes to which the immune system has been exposed increases, and thus the child becomes more likely to be protected against any new infections [29].

The results of the present study in placebo recipients suggest that it is not only memory of previous infections but also that the repertoire of concurrent infections (premunity) may determine the specificity of effective immune mechanisms against new infections [30]. It appears that the process of acquisition of premunity is probably highly dependent on the degree of endemicity and on the number of infective bites an individual receives. Immunologic cross-protection between different genotypes may also increase the acquisition of immunity.

In contrast to findings in the placebo recipients, the number of infections in vaccine recipients with clinical malaria was very similar to that in asymptomatic vaccine recipients. Vaccination with SPf66 had apparently eliminated the effect of multiple infections. This may be explained by differences in the course of an infection in vaccinated children, in whom the acquisition of multiple infections may be slower and the elimination of infections seems to be faster than in placebo recipients. But if immunity against individual genotypes takes some time to develop, the reduced duration of infections could interfere with the development of premunity. Presumably, this must be compensated for by the vaccine-induced protective effect to control high-density parasitemia.

The possibility that the vaccine is specifically more active against certain genotypes and that other genotypes gradually accumulate must therefore still be considered, although to date no significant differences in MSA2 genotype frequencies have been observed (data not shown). Due to the large polymorphism of MSA2, identification of a shift in allele frequencies at this locus would require large sample sizes. The possible selection for resistant genotypes in a vaccinated population must be considered and should be a priority question for further investigation, which also should include additional polymorphic markers.

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